PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Mar Tormo Ana M. Tari Gabriel Lopez-Berestein	§ § § §	
	§	Group Art Unit: 1805
Serial No. 08/726,211	§	
	§	Examiner: R. Schwartzman
Filed: October 4, 1996	§	
	§	Atty. Dkt.: UTXC:504/COD
For: INHIBITION OF BCL-2 PROTEIN EXPRESSION BY LIPOSOMAL ANTISENSE OLIGODEOXYNUCLEOTIDES	§ § § §	

DECLARATION OF GABRIEL LOPEZ-BERESTEIN AND ANA M. TARI UNDER 37 C.F.R. §1.131

WE, DR. GABRIEL LOPEZ-BERESTEIN AND DR. ANA M. TARI DECLARE AS FOLLOWS:

1. We, along with Dr. Mar Tormo, are co-inventors of the subject matter of the captioned patent application USSN 08/726,211.

2. It is our understanding that the Examiner in charge of the captioned application has rejected claims 1, 2, 5 and 6 as being anticipated by the abstract of Tormo *et al.* abstract #1190, *Proceedings of the American Association for Cancer Research*, vol. 37 pg 173, March 1996, and has rejected claims 1 and 2 as anticipated by the abstract of Almazan et al. abstract #2407, *Proceedings of the American Association for Cancer Research*, vol. 37 pg 353, March 1996.

3. Our invention was made and tested in this country prior to March 1996 and thus prior to the publication of the cited abstracts.

4. The fact that our invention was made and tested in this country prior to March of 1996 is evidenced by studies set forth in the attached notebook pages 280-288 of the laboratory notebook of Mar Tormo. Among other things, this notebook sets forth the following studies which exemplify the practice of our invention:

a) measurement of expression of Bcl-2 in Johnson, Daudi, Raji and Jurkat cells (p. 280);

b) a test of growth inhibition of the cell lines with liposomal AS (antisense), C (control) and B_2 oligonucleotides (p. 280-281);

c) graphs depicting apoptotic index of cells and cell density after treatment with control and antisense oligonucleotides (p. 282);

d) a repeat of the study of measurement of expression of Bcl-2 in Johnson, Daudi, Raji and Jurkat cells (p. 282-283);

e) growth inhibition studies of Johnson, Daudi, Raji and Jurkat cells in the presence of antisense and control liposomal oligonucleotides.

Each of items a) through e) as represented in the attached notebook were carried out in this country prior to March 1, 1996.

5. All statements made in this Declaration of my own knowledge are true and all statements made in this Declaration on information and belief are believed to be true, and these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both under 18 U.S.C. §1001 and may jeopardize the validity of this application or any patent issuing thereon.

1-6-98
Date

Gabriel Lopez-Berestein

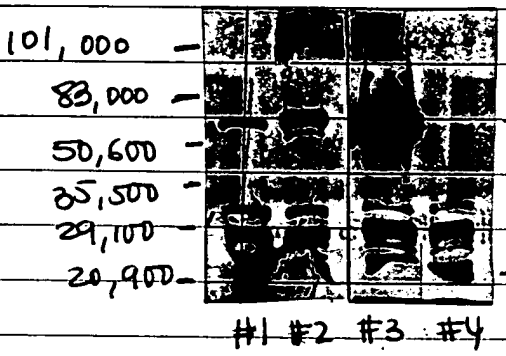
1-7-98
Date

Ana M. Tari

Sample	diluted 1/2 with 2x SB	µl for 5 µg	SB for 20 µl
Daudi	3.042	8.2 µl	11.8 µl
Johnson	3.104	8.05 µl	11.95 µl
Jurkat	4.712	5.3 µl	14.7 µl
Raji	4.651	6.4 µl	14.6 µl

⇒ Blotting:

p53 : 1/1000 (2d antimouse 1/4000)
Bcl-2 : 1/1000 (2d antirabbit 1/4000)



#1 Johnson
#2 Daudi
#3 Raji
#4 Jurkat

Wed

Monday 18 (1) Growth inhibition exp # 26

⇒ Test all cell lines with AS, C and B2 Rips in liposomes
Cell density do = 10,000/well in 100µl
Doses: 2, 3, 4, 5, 6, 7 and 8µM
⇒ Sonicate liposomes 12' and measure

(AS) (Nanop distribution) nm

	Peak 1	Peak 2	Fit Error 44.3
VOL :	36.3 (56%)	241.8 (44%)	Residual 0.00
INT :	36.8 (41.6%)	238.4 (58.4%)	
NUM :	34.7 (99.7%)	231.5 (0.3%)	

(C)

VOL	35.3 (55.8%)	172.2 (44.2%)	Fit Error : 39.98
INT	35.8 (34.6%)	170.7 (65.4%)	Residual : 0.00
NUM	33.8 (99.8%)	104.8 (0.7%)	

B213

SB for (1x)
20 µl
11.8 µl
11.95 µl
14.7 µl
14.6 µl

VOL: 29.5 (56.9%) H0.2 (43.1%) Fit Error 23.14
INT: 29.4 (30.6%) 108.7 (69.4%) Residual 50.39
NVH: 28.0 (98.5%) *

→ Add dyes to the cells. Incubate 5 day (day 5 → Saturday 23)

House 1/4000
Vaseline 1/4000

(2) Freeze Jurkat and Daudi cells (Alice P#2)
Jurkat → 7 tubes (with 4×10^6)
Daudi → 9 " (with 3×10^6)

at Wednesday 29 (1) Prepare new dyes: 40 nmoles of dyes with 400 nmoles of lipids

AS: $\frac{40 \times 10^3 \text{ pmoles}}{1270 \text{ pmoles}/\mu l} \rightarrow 31.5 \mu l$

C: $\frac{40 \times 10^3 \text{ pmoles}}{662 \text{ pmoles}/\mu l} \rightarrow 60 \mu l$

AS, C and

Lipids: $\frac{0.4 \text{ µmoles} \times 786.12 \text{ µg/µmole}}{30 \text{ µg}/\mu l} \rightarrow 10.5 \mu l$

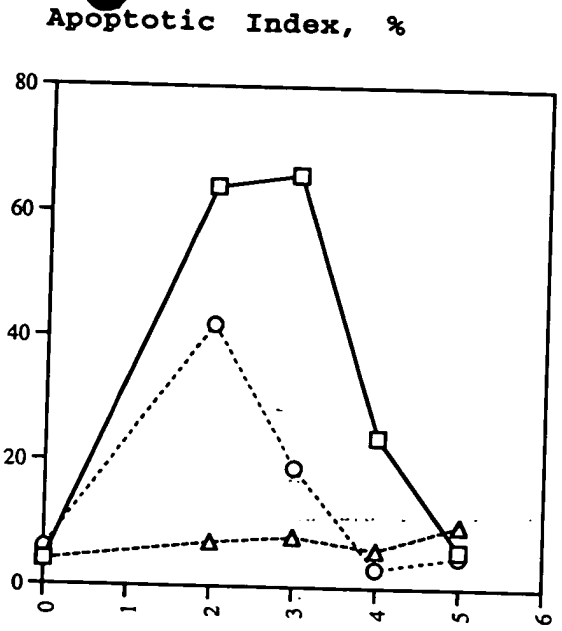
[Well in 100µl]
8µM
and measure:

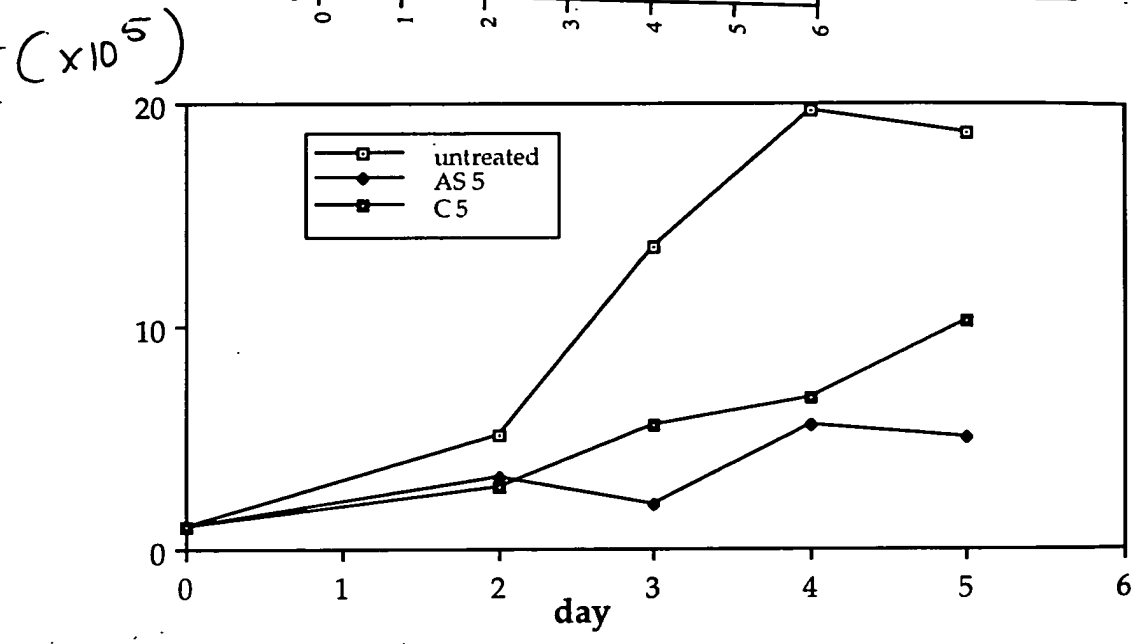
	AS	C	Empty
Lipids	10.5	10.5	10.5
Dye	31.5	60	Ø
t-butanol	2ml	2ml	1.5ml

nm
Fit Error 44.3
Residual 0.00

(*) From pag 276 →

Fit Error: 39.98
Residual: 0.00





204,000 –
121,500 –
82,000 –
50,200 –
34,200 –
28,100 –
19,400 –
7,300 –

12/24

(2) I will go to TUU again the gel with extract of the four cells lines untreated for to test the bcl-2 expression because in the previous blott there are many inespecific bands and a bad transferance.

Sample	diluted 1/2 with 2x SB	µl for 25µg	µl for 50 µg
Johnson	3.104	8.1 µl	16.2 µl
Daudi	3.042	8.2 µl	16.4 µl
Jurkat	4.712	5.3 µl	10.6 µl
Raji	4.651	5.4 µl	10.8 µl

1. Johnson 5
2. Daudi 6.
3. Jurkat 7.
4 Raji 8.

25μg 50μg

12/24/95 Growth inhibition exp # 26. day 5
Alamar Blue Assay.
Result:
(1) Johnson

	2	3	4	5	6	7	8	∅	Blank		
AS	0.762	0.725	0.108	-0.164	-0.241	-0.242	-0.249	0.792	-0.244		
	0.761	0.624	-0.035	-0.213	-0.250	-0.261	-0.250	0.742	-0.247		
C	0.726	0.721	0.705	0.530	0.196	-0.104	-0.229	0.736			
	0.725	0.788	0.729	0.625	0.327	-0.175	-0.218	0.764			
B_{29}	0.788	0.758	0.736	0.794	0.634	0.329	0.124	0.721			
	0.776	0.784	0.732	0.739	0.453	-0.053	-0.090	0.764			

	AS	C	B_2 (% viability)
2	100%	96%	104%
3	89%	100%	102%
4	5%	95%	97%
5	0%	76%	101%
6	0%	35%	72%
7	0%	0%	18%
8	0%	0%	2%

(2) Raji:

284

	2	3	4	5	6	7	8	∅	Blk		
AS	0.832	0.828	0.832	0.869	0.680	0.392	0.370	0.812	-0.249		
	0.832	0.850	0.758	0.786	0.725	0.637	0.367	0.847	-0.259		
C	0.828	0.883	0.799	0.807	0.798	0.695	0.245	0.828			
	0.773	0.816	0.747	0.730	0.830	0.683	0.353	0.847			
B_2	0.823	0.846	0.823	0.835	0.866	0.872	0.817	0.815			
	0.878	0.813	0.808	0.816	0.711	0.647	0.741	0.813			

	AS	C	B_2
2	100%	97%	103%
3	101%	100%	100%
4	96%	98%	98%
5	100%	93%	99%
6	85%	98%	95%
7	62%	83%	92%
8	44%	36%	94%

(% viability)

(3) Jurkat:

	2	3	4	5	6	7	8	∅	Blk		
AS	0.780	0.787	0.736	0.268	-0.107	-0.217	-0.233	0.795	-0.245		
	0.787	0.751	0.617	0.076	-0.111	-0.241	-0.245	0.825	-0.249		
C	0.784	0.780	0.715	0.629	0.334	-0.082	-0.240	0.788			
	0.808	0.780	0.749	0.603	0.225	-0.205	-0.233	0.791			
B_2	0.856	0.796	0.772	0.762	0.705	0.722	0.574	0.785			
	0.804	0.761	0.817	0.752	0.757	0.664	0.579	0.801			

	AS	C	B_2
2	98%	100%	104%
3	96%	98%	97%
4	85%	92%	99%
5	33%	77%	95%
6	0%	35%	91%
7	0%	0%	87%
8	0%	0%	72%

(% viability)

viability

(4) Daudi :

	2	3	4	5	6	7	8	Ø	Bluk		
AS	0.460	0.351	0.123	-0.202	-0.219	-0.222	-0.219	-0.481	-0.227		
	0.533	0.352	0.225	-0.215	-0.221	-0.217	-0.220	0.498	-0.229		
C	0.489	0.463	0.337	0.076	-0.203	-0.213	-0.222	0.516			
	0.503	0.451	0.281	0.170	-0.148	-0.220	-0.215	0.510			
B_2	0.514	0.463	0.419	0.387	0.238	-0.099	-0.103	0.458			
	0.498	0.461	0.470	0.388	0.289	-0.021	-0.144	0.520			

	AS	C	B_2
2	97%	87%	89%
3	62%	80%	81%
4	39%	54%	78%
5	0%	21%	68%
6	0%	0%	46%
7	0%	0%	0%
8	0%	0%	0%

(% viability)

1/5/10(1) WED Exp #6

(1) To test if there are bcl-2 inhibitors with AS doses: 2, 3, 4, 5, 6 and 7 μM of AS and C drugs

(2) Prepare the drugs like always and sonicate for 12' (4'-4'-4')

(3) After measurement of the hydrodynamic size:

AS

F-ANN UPTAKE
Menu File: C370.TBL 1/3/96

Run time	Data Ch	tensity	In	Sensitivity
0 Hours 7 Mins 48 Secs	131.7K	1.5kHz	138	255

Gaussian Analysis			(Vesicl	Distribution Analysis			
	Mean Dia	Std Dev	Chi Sq	Ba	Peak 1	Peak 2	Peak 3	
VOL:	152.3 nm	112.7 nm	297.42		12.5 %	35.5	192.0	Fit Error
		74.0 %			5.4 %	54.1 %	40.5 %	5.813
INT:	133.2 nm	98.5 nm	297.42		12.7 %	36.1	176.8	Residual
		74.0 %			1.3 %	35.7 %	63.1 %	51.909
NUM:	24.8 nm	18.3 nm	297.42		29.1	169.6	-----	
		74.0 %			99.4 %	0.6 %	-----	

C

F-ANN UPTAKE
Menu File: C370.TBL 1/3/96

Run time	Data Cntensity		Sensitivity
0 Hours 9 Mins 24 Secs	37.9K	.4kHz	255

Gaussian Analysis			(Vesic	Distribution Analysis			
	Mean Dia	Std Dev	Chi Sq	B	Peak 1	Peak 2	Peak 3	
VOL:	76.0 nm	51.3 nm	4.44		26.0 %	80.7	-----	Fit Error
		67.5 %			30.1 %	69.9 %	-----	15.788
INT:	87.2 nm	58.8 nm	4.44		27.9	81.7	-----	Residual
		67.5 %			14.6 %	85.4 %	-----	47.565
NUM:	18.3 nm	12.3 nm	4.44		21.4	63.4	-----	
		67.5 %			92.1 %	7.9 %	-----	

(4) Add to the cells (Johnson's cells plated in a 24 well plate at the 100,000 cells/well, 1 ml in each well)

(5) Incubate for 3 days.

(2) Check again the levels of my four cell lines (Cu. perius blot it was very dirty). Prepare new supernatant of Raji and Jurkat cells and measure the amount of protein again.

Curve Fit: Linear — Corr. Coeff: 1.00
Equation: y = A + B * x — Std Units: ug/ul
A = 0.00769 B = 0.287

PLATE BLANK			Well	OD	Mean	Std Dev	CV
BL			A12	0.053	0.053	*****	*****

STANDARDS	Value		Well	OD	Mean	Std Dev	CV
STD01	0.000	ug/ul	A1	0.017	0.012	0.005	38.19
			B1	0.008			
			C1	0.011			
STD03	0.500	ug/ul	A3	0.186	0.144	0.050	34.57
			B3	0.156			
			C3	0.089			
STD04	0.750	ug/ul	A4	0.398	0.224	0.157	70.21
			B4	0.182			
			C4	0.092			
STD06	1.500	ug/ul	A6	0.588	0.440	0.131	29.70
			B6	0.389			
			C6	0.342			

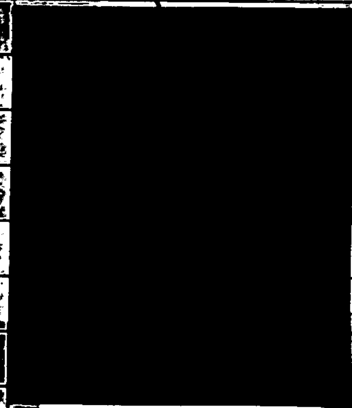
UNKNOWNS	Mean	Std Dev	CV	Well	Value	OD	Dil.Factor
DAUDI	1.940	0.264	13.61	E3	1.754	0.058	10.00
				F3	1.824	0.060	
				G3	2.243	0.072	
JOHNS	1.754	0.160	9.108	E1	1.929	0.063	10.00
				F1	1.615	0.054	
				G1	1.720	0.057	
JURK	1.603	0.053	3.322	E2	1.615	0.054	10.00
				F2	1.650	0.055	
				G2	1.545	0.052	
RAJI	2.836	0.126	4.434	E4	2.731	0.086	10.00
				F4	2.801	0.088	
				G4	2.975	0.093	

Then diluted with 1/2 x SB for 25 µg

Lane	Sample		
1	Johnsy	0.877	40 µl
2	Daudi	0.97	36 µl
3	Jurkat	0.802	44 µl
4	Raji	1.420	25 µl.

Blott 1° (Bd-2) : 1/1000 (S)
2do : 1/4000

(1) Exposure time = 30"



#1 #2 #3 #4

1. Johnson
2. Daudi
3. Jurkat
4. Raji

35 µg total protein

1/8/96 WB exp #6

1) Count the cells manually:

ϕ = 1.065 x10^6 (99% viab.)
2AS = 0.845 x 10^6 (99%)
3AS = 0.580 x 10^6 (91%)
4AS = 0.325 x 10^6 (92%)
5AS = 0.555 x10^6 (89%)
6 AS = 0.170 x 10^6 (55%)

2C = 1.205 x 10^6 (96%)
3C = 0.975 x 10^6 (98%)
4C = 0. 645 x 10^6 (97%)
5C = 0.385 x 10^6 (94%)
6C = 0. 190 x 10^6 (76 %)

2) Lyse the cells (protocol)
3) Normalization of amount of protein
) Run the gel
4) Transfer O/N 33 volt

PLATE BL
BL
STANDARD
STD01
STD02
STD03
STD04
STD05
STD07
UNKNOWNS
0
AS2
AS3
AS4
AS5
AS6
C2
C3
C4
C5
C6